SEX SPECIFIC DIFFERENCES IN THE GENOMIC LANDSCAPES OF NAÏVE MALE AND FEMALE $CD8^+$ T-CELLS

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ABSTRACT

Sex specific differences in the immune response have been demonstrated in organisms from sea urchins to humans. In mammals, these differences have been linked to processes in both the innate and adaptive immune responses, and many autoimmune disorders (e.g. systemic lupus erythematosus, rheumatoid arthritis, thyroid disease) show a bias towards developing in females. However, the complex mechanisms behind these discrepancies are not fully understood. $CD8^+$ T-cells are a subtype of lymphocytes that are critical to the production of and response to inflammatory signaling, so they are likely to play a role in these autoinflammatory processes. Previous work in murine naïve $CD8^+$ T-cells has shown that naïve female $CD8^+$ T-cells follow different patterns of differentiation after antigen exposure than male $CD8^+$ T-cells. Both sexes form the same number of differentiated cells, but the ratio of T-cell subsets formed differs between males and females. Female $CD8^+$ T-cells tend to differentiate into short-lived effector cells (SLECs) that respond directly to invaders and produce large amounts of inflammatory cytokines, while their male counterparts tend to differentiate into memory precursor effector cells (MPECs) that respond less directly to infection but can transition into the pool of cells that forms immunological memory. Co-transfer experiments and assays of T-cell function suggest that this altered differentiation may be cell intrinsic, as opposed to being a product of different niches between males and females. To investigate the potential genomic

factors driving sex specific differences in $CD8^+$ T-cell differentiation, I performed RNA-seq and PRO-seq on naïve male and female $CD8^+$ cells. These data revealed a potential role for the Type I Interferon response and the related transcription factor IRF7 in the differentiation of female naïve $CD8^+$ T-cells. Future investigations will be required to elucidate the exact mechanism of this differential expression of the Type I IFN pathway.

BIOGRAPHICAL SKETCH

Katrina Haught is originally from New Milford, CT. She attended Stony Brook University for her undergraduate studies and obtained a BS in biochemistry. During her time at SBU she conducted research in several different laboratories. In the Scarlata group, Katrina helped investigate the effects of hypoosmotic stress on the colocalization of G-protein subunits and PLCβ in cardiac cell caveolae. She also worked on a summer project at Cold Spring Harbor Laboratory with Dr. Leemor Joshua-Tor, investigating the interactions between the protein Argonaute and one of its binding partners, GW182. Katrina's undergraduate honors thesis was written on work done in the Czaplinski laboratory at SBU, where she looked at how back signaling in pre-synaptic neurons contributes to localization of mRNA translation.

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More generally, I would like to express gratitude to all of the members of the Grimson lab, both for their assistance in scientific matters and their friendship. The atmosphere in the lab is always friendly, collaborative, and generally fun, making my time there much more enjoyable.

Finally, I would like to thank my mom, who always stressed to me the importance of a higher education. Without her passion and support I would not be where I am today.

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Background and Significance

The immune system allows organisms to defend themselves from both outside invaders and malignant events inside of the body. Two branches of this system, innate and adaptive immunity, work together to provide this protection. The innate immune response, whose blueprint extends back to single celled organisms, takes advantage of conserved marks from invaders (pathogen associated molecular patterns, or PAMPs) to mount a general response to injury or infection.[1,2] In contrast, the adaptive immune response, which is responsible for immunological memory, is comprised of specialized cells that generate antigen specific receptors, allowing them to mount a more specialized assault.[3,4] These systems cross-regulate each other extensively in vertebrates through direct cell-cell interactions, cytokine production, and other signaling mechanisms.[5] This text will focus on one of the key players in the adaptive immune response, cytotoxic $CD8^+$ T-cells, and the sex specific differences between naïve $CD8^+$ T-cells from males and females.

$CD8^+$ T-cells are a specific subset of leukocytes that are formed in bone marrow (or the fetal liver) and then go through an extensive maturation process in the thymus.[6] Their main function is to guard against pathogens that invade healthy cells by releasing cytolytic factors that can kill infected cells (e.g. granzyme B, perforin) and producing cytokines that can activate other immune cells from both the innate and adaptive branches.[7,8] Aberrant activation of these destructive measures would be harmful to the organism, so $CD8^+$ T-cells require three separate signals for optimal initiation. First, T-cell

receptors (TCRs) must recognize an antigen presented on the surface of another cell by major histocompatibility complexes (MHCs). Class I MHCs, which interact specifically with $CD8^+$ T-cells, are assembled in the endoplasmic reticulum and loaded with peptides derived from protein degradation pathways in the cytoplasm.[9] Along with this TCR:MHC I interaction, CD28 on the T-cell must interact with a B7 ligand (CD80/CD86) on the antigen presenting cell.[10,11] Finally, the effects of these two activated pathways are amplified by the presence of pro-inflammatory cytokines, such as Il-12 and Type I interferons (Type I IFNs).[12]

$CD8^+$ T-cells accomplish the targeted destruction of presented antigens through the specificity of their TCRs, which possess variable regions formed by somatic recombination that can recognize a large spectrum of ligands.[13] Each T-cell expresses its own TCR that recognizes a specific ligand, and the fate of the T-cell is dependent on their TCR binding to MHC proteins with the appropriate affinity (positive selection) and not recognizing self-antigens presented in the cortex of the thymus (negative selection).[14] After positive and negative selection of T-cells, there are very few cells left in the naïve $CD8^+$ pool that will respond to any given antigen.[15] So, T-cells that can recognize antigens currently in circulation must rapidly expand their population to respond to the infection or malignancy.[16] Once the threatening antigen has been withdrawn, most of the responding $CD8^+$ population will die off within ~60 days, leaving a small subset of memory T-cells to react to the same antigen in the future.[17,18]

Investigations into what differentiates $CD8^+$ T-cells that die after antigen exposure from those that live on in the memory pool has led to the discovery of several different $CD8^+$ T-cell subsets. Before antigenic stimulation occurs, some naïve $CD8^+$ T-cells upregulate the cell surface markers CD44 and CD122 that are characteristic of memory T-cells, forming a distinct group from true naïve $CD8^+$ T-cells (memory phenotype cells, MPs).[19,20] Activated T-cells can act as effectors that release cytolytic factors to directly attack infected cells and then die, or as memory cells that outlive the immunological challenge and allow the organism to respond more rapidly to later exposure to the same antigen. This distinction between effector and memory T-cells is not an all-or-nothing dichotomy. Instead, the activated population is comprised of a diverse spectrum of T-cell subsets that fulfill different roles between fully effector and fully memory functions.[21] Two subtypes of effector T-cells that can be distinguished based on Il-7 receptor alpha (CD127) and Killer Cell Lectin Like Receptor G1 (KLRG1) expression are short lived effector cells (SLECs, $CD127^{lo}$ $KLRG1^{hi}$) and memory precursor effector cells (MPECs, $CD127^{hi}$ $KLRG1^{lo}$).[21–23] SLECs and MPECs both produce cytokines and can induce apoptosis in target cells, but MPECs are less cytolytic and are more likely to become memory T-cells.[24,25]

Because of the functional disparity between SLECs and MPECs, the ratios of each subtype formed by naïve $CD8^+$ T-cell activation would influence both the strength of the cytokine response and the development of the memory T-cell pool. The ratio of SLECs to MPECs is influenced by cell

extrinsic factors, such as inflammatory cytokines (Il-12, Type I IFNs) and costimulation by helper $CD4^+$ T-cells.[26,27] Our group has elucidated several examples of cell intrinsic factors that can influence this SLEC:MPEC ratio. Formation of immunological memory is the basis of vaccine efficacy, and investigation of the dynamics of memory T-cell formation in newborns is critical to understanding and improving vaccine delivery. Several studies in mice have investigated the different expansion ratios, gene expression, and chromatin arrangement of adult derived $CD8^+$ T-cells versus their neonatal counterparts.[28–33] Adult T-cells, produced in the thymus, are more proficient at forming memory T-cells, while neonatal T-cells derived from the liver form a higher proportion of SLECs, release a different set of cytokines, and respond more rapidly to infection.[28,33] Additionally, before antigen stimulation and expansion, naïve neonatal $CD8^+$ T-cells form a larger population of $CD44^+CD122^+$ virtual memory (VM) cells (a subset of MP cells), where adult cells have a larger true naïve population.[33–35]

It is important to note that the differences discussed in previous studies are not due to different niches in adults versus neonates and are instead cell intrinsic, since transfer of neonatal cells to adults does not cause them to expand in the same way as an adult population. Neonatal $CD8^+$ T-cells express higher levels of transcription factors associated with effector cells (e.g. T-bet, BLIMP1), while adult cells express higher levels of transcription factors related to memory cell development (e.g. Eomes).[28] The expression of certain microRNAs, such as miR-29, miR-130, and miR-150, has also been shown to

modulate T-cell population expansion into different subtypes.[29,30,36] Loss of miR-150 severely attenuates $CD8^+$ T-cell expansion, interfering with the development of cytotoxic effector cells.[30] miR-29 (as well as miR-150) is a negative regulator of the T-cell infection response and is downregulated in neonatal cells. On the other hand, miR-130 is upregulated in neonates and is connected to T-cell activation.[29] Both miR-29 and miR-130 target important regulators of T-cell fate determining pathways, including Eomes, T-bet (both targeted by miR-29), and IRF1 (targeted by miR-130).[29] Lin28b, a negative regulator of the developmentally critical miRNA let-7, is more highly expressed in neonatal cells.[31] Forced expression of Lin28b in adult $CD8^+$ T-cells causes them to form a large population of MP cells in a manner similar to neonatal populations.

Another interesting immunological variable affecting $CD8^+$ T-cell development that intersects with age is sex.[37] It has been well established that the immune response differs between the sexes, with females mounting a more effective assault against most infections and cancerous cells.[38,39] These sex specific differences in the immune response extend from sea urchins up to humans.[40] Many important genes involved in the innate and adaptive immune responses, including pattern recognition receptors (e.g. TLR7, TLR8) and cytokine receptors, are found on the X chromosome and differentially expressed in males and females.[41,42] miRNAs, some of which are enriched on the X chromosome, have also been shown to modulate immune responses, although immune regulation is not limited to these X chromosomal

miRNAs.[43,44] Immune cells in females, including dendritic cells (DCs) and $CD8^+$ T-cells, release higher levels of pro-inflammatory cytokines.[39,45] B-cells, another type of adaptive immune cells that produce antibodies, are also more active in females, spawning higher amounts of immunoglobulin.[39]

However, this increased efficiency at responding to immunological threats comes with a high cost. While the negative selection of T-cells in the thymus is designed to screen out lymphocytes that respond inappropriately to self-antigens, this process is not perfect. A slew of serious medical issues, including asthma, type 1 diabetes (T1D), systematic lupus erythematosus (SLE), rheumatoid arthritis (RA), and thyroid disease are at least partially caused by immune cells responding inappropriately to harmless self-antigens.[40] Females are far more prone to developing most common autoimmune disorders besides T1D.[40,44,46] Eighty percent of autoimmune disorder patients are women, and these diseases are a leading cause of death for women from puberty onward.[39,47] Some autoimmune disorders, such as Hashimoto's hypothyroidism and SLE, are influenced by cycles in the expression of female sex hormones, and disorders that affect female sex organs (e.g. polycystic ovary syndrome) show a high comorbidity with autoimmune disorders.[48–50] Autoimmune disorders are not only debilitating, but they are also estimated to cost residents of the US alone over $100 billion dollars a year in direct healthcare costs.[51] Because of this humanistic and economic burden, elucidating the mechanisms behind these sex specific differences is an important research question.

Several factors influencing the differential immune response between males and females have been identified. Hormones that are differentially expressed between the sexes, such as estrogen, progesterone, and the androgens, all influence immune function.[39] There are 2 types of estrogen receptors, ERα and ERβ, and they are differentially expressed in various immune cell types.[52] Estrogen increases the strength of the $CD8^+$ T-cell response, production of antibodies by B-cells, and the expression of pattern recognition receptors in innate immune cells.[53] It also plays a role as a direct stimulator of the Type II IFN IFNγ, which encourages inflammation.[54] The transcription factor AIRE is a key component in negative selection, mediating the expression of the self-antigens used to screen T-cells for harmful autoimmune responses.[55] Expression of AIRE is downregulated by estrogen.[56] This downregulation may lead to the survival of more autoreactive T-cells, as is the case for AIRE deficient mice.[55] Progesterone, which is expressed at various points during the menstrual cycle and pregnancy, can alter the signaling of multiple innate and adaptive immune cell types and is generally anti-inflammatory.[38,57] The male associated sex hormones, including testosterone, suppress expression of IFNγ, Il-4, and Il-5, and dampen the immune response.[40] Because of the importance of hormones as cell-extrinsic signals for male and female cells, it is critical to study the differences between them in an environment that provides the same external signaling to both

males and females. This can be achieved through *in vitro* experiments, or through transfer techniques that will be discussed later.

Known factors that influence the sex specific differences in the immune response extend more generally to the expression of genes on the X chromosome. While X chromosome inactivation generally prevents the expression of the same genes on the two copies present in females, this process is not perfect and some genes in humans (and to a lesser extent, mice) escape inactivation.[58,59] $CD4^+$ T-cells from female SLE patients express higher levels of specific genes on the X chromosome than healthy females or males with SLE.[42] Alterations of sex chromosome ratios in genetic disorders have also supported the hypothesis that a higher number of X chromosomes will lead to the more autoinflammatory responses normally seen in females. Turner's syndrome describes the situation where only one X chromosome is present without a Y chromosome (X0). Turner's syndrome patients show reduced immunoglobulin production, an attenuated lymphocyte response to antigens, and a lower propensity to develop SLE, all in line with a more male-like response than XX females.[60,61] The opposite is true for Klinefelter's syndrome (XXY) males, who show a higher propensity for developing autoimmune disease than XY males.[62]

Considering the previously discovered roles for miRNAs in immune cell development and function, it is not surprising that differences in miRNA expression also play a role in sex specific differences. For example, miR-29 is flanked by putative estrogen response elements, and is selectively

downregulated in the frontal cortex of female mice upon exposure to radiation.[63,64] Other miRNAs, including the miR-182-96-183 cluster, miR-31, miR-155, miR-127, and miR-379, are upregulated in female NZB/W_{F1} mice (a model for SLE) as opposed to male NZB/W_{F1} mice, and all of these miRNAs besides miR-155 are expressed more strongly after treatment with estrogen.[65] The miR-182-96-183 cluster and miR-31 have been implicated in oncogenesis, while miR-31 has also been shown to influence T-cell development through upregulating Il-2 signaling.[66–68] BCL6, a transcription factor involved in the differentiation of $CD4^+$ T-cells, is targeted by miR-127.[69] The immunomodulatory effects of miR-379 are not well understood at this time.[65] On the other hand, miR-155 was one of the first miRNAs characterized, based on its oncogenic properties in B-cells.[70] Activated $CD8^+$ T-cells upregulate miR-155, effector T-cells express higher levels of miR-155 than memory cells, and this expression of miR-155 is required for response to viruses and cancer cells.[71,72]

Sex specific differences in the immune system are certainly important to consider both for their clinical importance, and the fact that research studies must take them into account to obtain valid experimental results.[41] However, the evolutionary reasoning behind these different responses still remains unclear. The aggressive immune response in females may seem counterintuitive, since females must carry non-self cells for an extended period during pregnancy, but it has been shown that increased levels of certain inflammatory cytokines (e.g. Il-6) during pregnancy decreases the likelihood of

recurring miscarriages.[73] Some of the first investigations into this quandary proposed a simple explanation based on two assumptions. The first was that since males invested more resources into attracting mates, they had to lose some advantages in the immune system. The second key tenant was that the main advantage of a strong immune response was increased longevity, and females (because of their larger time investment in offspring) stood more to gain from extending their lifespan than males.[74] This viewpoint has been challenged as over simplistic, with counter arguments pointing out that longevity is not as directly correlated with a stronger immune response as these ideas would suggest because of autoimmune complications and non-fatal infections.[75] It has been suggested that females may produce higher levels of immunoglobulins because newborns depend heavily on the antibodies inherited from their mother during the first few weeks of life.[76] In any case, the evolutionary drive behind the more aggressive female immune response is still an important area of current investigation.

While many general mechanisms for sex specific differences in the autoimmune response have already been described, there are still important signaling pathways to be explored that are involved in these responses. For example, what immunological pathways mediate the stronger $CD8^+$ T-cells response mounted by females, and are they cell intrinsic or extrinsic? Prior work by our collaborators has shown that naïve male and female $CD8^+$ T-cells from mice show different patterns of expansion when exposed to antigen. During expansion, the T-cells from each sex form roughly the same number of

differentiated cells, but the ratios of effector versus memory subtypes differ. Females tend to form a higher proportion of the more inflammatory SLECs, while males have a higher proportion of MPECs. Additionally, female $CD8^+$ T-cells produce higher levels of IFNγ and the cytolytic factor granzyme B upon exposure to the inflammatory cytokine Il-12. A series of adaptive co-transfer experiments were performed to determine whether these properties were due to a different immunological niche for $CD8^+$ T-cell expansion in each sex. Equal numbers of male and female naïve $CD8^+$ T-cells responsive to a specific antigen were transferred into a male mouse, and it was found that even in the male niche, female cells still showed a preference for differentiating into SLECs vs MPECs (Yee Mon, *unpublished data*).

Based on these previous results in male and female $CD8^+$ T-cells, we became interested in discovering what about the naïve female $CD8^+$ T-cells made them different from the males. There were no obvious phenotypic differences between male and female $CD8^+$ T-cells in adaptive co-transfer experiments where the cells were not exposed to antigen, so we decided to use a genomic approach to evaluate the naïve cells. We performed RNA-seq and PRO-seq on naïve $CD8^+$ T-cells from males and females and found that the Type I IFN response pathway was upregulated in female cells. This pathway is critical to the autoimmune response, and discovery of its upregulation in female $CD8^+$ T-cells both informs approaches to treating autoimmune disorders, and underscores the importance of designing future experiments to be mindful of this split between males and females.

Materials and Methods

RNA-seq library preparation and analysis

Naïve $CD8^+$ T-cells were isolated from 9-week-old B6 x gBI mice using flow cytometry (sorted to select a population that was $CD8^+CD4^-Vb8^+Va2^+$) and provided to us by Kristel Joy Yee Mon (Rudd Laboratory, Cornell University). 200,000 cells from n=3 males and n=3 females were immediately placed into Trizol after sorting. Ribosomal RNA depleted libraries were generated using 88-100ng of RNA by the RNA Sequencing Core (Cornell University). The resulting libraries were sequenced on an Illumina NextSeq500, and aligned to the mm10 genome using TopHat.[77] Differentially expressed genes were determined using cuffdiff2, with an FDR cutoff of ≤ 0.05.[78,79]

PRO-seq library preparation and analysis

PRO-seq libraries were made from naïve $CD8^+$ T-cell nuclei from n=2 males and n=2 12-week-old B6 x gBI mice provided by the Rudd laboratory.[80,81] Cells were permeabilized in buffer P (100 mm Tris-Cl, pH 7.5, 10mM KCl, 250mM sucrose, 5mM $MgCl_2$, 0.5mM $CaCl_2$, 1mM EGTA, 0.05% Tween-20, 1x EDTA free protease inhibitor (Thermo Scientific), 0.5mM DTT, 40 units RiboLock RNase inhibitor) for 10 minutes on ice, then washed in buffer W (10mM Tris-Cl pH 8.0, 300mM sucrose, 10mM NaCl, 2mM $MgAc_2$, 0.008% Tween20, 0.5mM DTT, 1x EDTA free protease inhibitor, and 40 units RiboLock RNase inhibitor), and finally flash frozen in buffer F (50mM Tris-Cl pH 8.3, 40% glycerol, 5mM $MgCl_2$, 0.1mM EDTA, 0.5mM DTT, and 40 units

RiboLock RNase inhibitor) using liquid nitrogen. Between each buffer exchange, cells were spun at 1000xg at 4°C for 8 minutes.

800,000 frozen nuclei in 50µL buffer F were used for each library. Library preparation mostly proceeded as previously described, with a number of new modifications.[80] The nuclear run-on was performed with 4 biotinylated nucleotides at a concentration of 15µM, and the sample was kept at 37°C for 5 minutes instead of 3 min. Additional washes with equal volumes of acid phenol: chloroform and chloroform were added after the Trizol RNA extraction. Ethanol precipitations were kept at -20°C for 1 hour, and then spun at 13000RPM at 4°C for 30 min. Base hydrolysis of the RNA by NaOH was performed on ice for 15 min. Mixing of the biotinylated RNA with streptavidin beads was carried out for 30 min. Instead of an RNA adaptor, a pre-adenylated 3' RNA adaptor (Pro3D, App-GATCGTCGGACTGTAGAACTCTGAAC-/3InvdT/) was used. RppH was used for the 5' cap and triphosphate repair, and an RNA adaptor (Pro5R, CCUUGGCACCCGAGAAUUCCA) was ligated to the enriched RNA. cDNA from each library was amplified at 13 cycles, based on results from a test amplification run on an 8% PAGE gel at 250V for 1.5 hours. The libraries were then PAGE purified (8% PAGE gel at 250V for 1.5 hours), with bands cut out for each library from 140bp to ~1000bp, and DNA was extracted from the gel by placing the gel pieces on a rotator at 50°C for 16 hours. Libraries were quantified using a Qubit 2.0 fluorometer, and then their size distributions were

assessed using an AATI Fragment Analyzer. Sequencing was performed on an Illumina NextSeq500 using single end 75bp reads.

Adaptors were trimmed from the reads using cutadapt, reads were filtered for quality and size ≥ 15 nucleotides, and then the reads were aligned to the *Mus musculus* mm9 genome using bowtie2.[82,83] The program featureCounts was used to perform read summarization for genes and promoter regions.[84] Transcriptional regulatory elements were identified using dREG.[85] TREs were called as promoters if they were located in the area -500 to +500bp around a known transcription start site. Differentially expressed genes and promoters were analyzed using EdgeR, with an FDR cutoff of ≤ 0.05.[86] After analysis of the resulting data, it was found that one of the female samples was very low quality (lower number of reads that aligned to the mouse genome, sporadic gene expression that did group together with the other samples, male or female), so this library was excluded from further downstream analyses. An additional set of PRO-seq libraries (n=3 males, n= 3 males) was prepared at the same time as the RNA-seq samples described above using 1 million cells per library, but these libraries were unusable due to low DNA yield and sequencing errors. Therefore, while analysis of the RNA-seq data used n=3 males and n=3 females, analysis of the PRO-seq data only uses n=2 males and n=1 females.

Gene ontology (GO) enrichment analysis and gene set enrichment analysis (GSEA)

Gene ontology enrichment analysis for the RNA-seq and PRO-seq samples was performed using GOrilla, with an FDR cutoff of 0.25.[87] The

background set used for the comparison was the total number of genes or promoters detected by each sequencing technique (n= 10519 genes for RNA-seq, n= 9006 promoters for PRO-seq). Since GO analysis can often lead to the return of multiple categories related to the same signaling processes (e.g. “response to interferon-alpha” versus “cellular response to interferon-alpha”), the program REViGO was used to compress together these redundant terms.[88]

Gene set enrichment analysis was performed using GSEA 3.0, including gene sets from GO terms, the MSigDB, and the ImmGen Consortium.[89,90] The genes or promoters identified during sequencing were ranked based on the metric (sign of the $\log_2$(fold change)) times the inverse of the adjusted P-value, and then compared to curated gene sets using GSEAPreranked.

Motif enrichment analysis

Motif enrichment analysis was performed using iRegulon, an app for Cytoscape.[91,92] The 10K motif collection was used, and the search was restricted to the area -500bp to +500bp around the TSS of differentially expressed genes from the RNA-seq data. Motifs with a normalized enrichment score greater than 3.0 were called as enriched in the differentially expressed genes, yielding 103 motifs and 17 motif clusters.

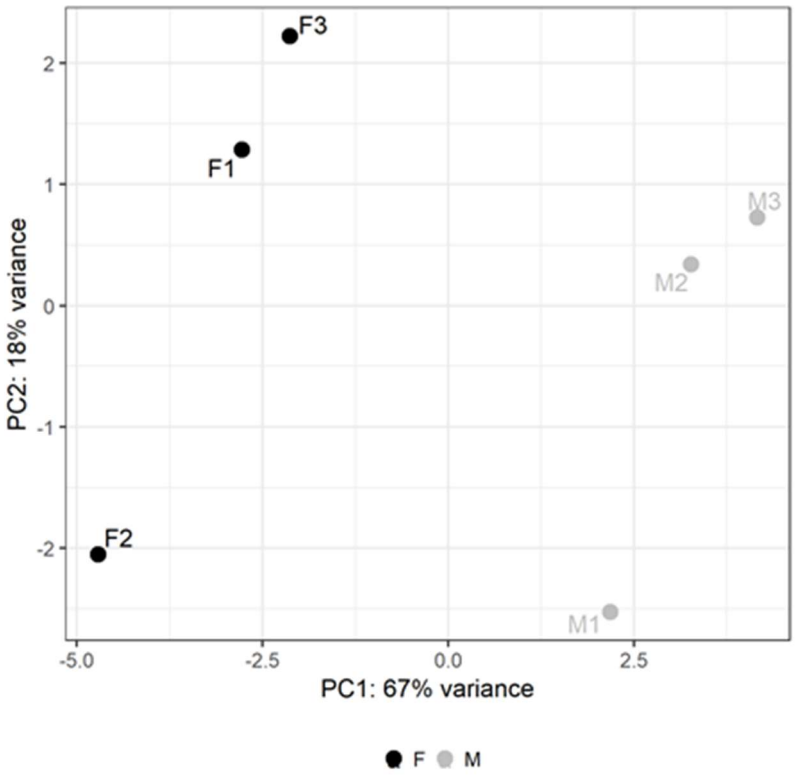
Results

Male and female naïve CD8⁺ T-cells show different patterns of gene expression

Naïve CD8⁺ T-cells ($CD8^+CD4^-Vb8^+Va2^+$) were isolated from 9-week-old B6 x gBI mice using flow cytometry. RNA-seq was performed to evaluate the gene expression patterns of male versus female CD8⁺ T-cells (Figure 1). PCA shows genome-wide differences between the male and the female cells that were greater than the differences between the replicates (Figure 1A), and this clustering of the sexes is supported by sample to sample distance clustering (Figure 1B). 50 genes were found to be differentially expressed between the male and female naïve CD8⁺ T-cells (FDR<0.05), with 38 genes upregulated in the female cells and 12 upregulated in the male cells. 18 of these genes have known functions in the immune system, with 17 upregulated in females and 1 upregulated in males. Genes with known immune functions and a fold change ≥1.5 are shown in Figure 1C. Notably, most of the members of the interferon induced proteins with tetratricopeptide repeat (Ifit) family were enriched in the female CD8⁺ cells, as well as other proteins involved in the Type 1 IFN response (e.g. Irf7, Mx1, Oasl2, Isg15).[93]

Figure 1: RNA-seq performed on naïve $CD8^+$ T-cells. N=3 males, 3 females. Genes were considered differentially expressed if they achieved an FDR ≤0.05 after cuffdiff2 analysis. A) Principal component analysis performed on the log transformed counts from each of the RNA-seq libraries. B) A sample to sample distance heatmap clustering of the libraries based on variance stabilized counts. C) Volcano plot highlighting differentially expressed genes involved in the immune response with a fold change ≥1.5. Xist, Eif2s3y, Ddx3y, and Kdm5d (genes expressed on sex chromosomes that are expected to be differentially expressed in most cell types) are excluded for clarity.

A.


F3
F1
M3
M2
F2
M1
2
1
0
-1
-2
-5.0
-2.5
0.0
2.5
PC2: 18% variance
PC1: 67% variance
F
M

B.

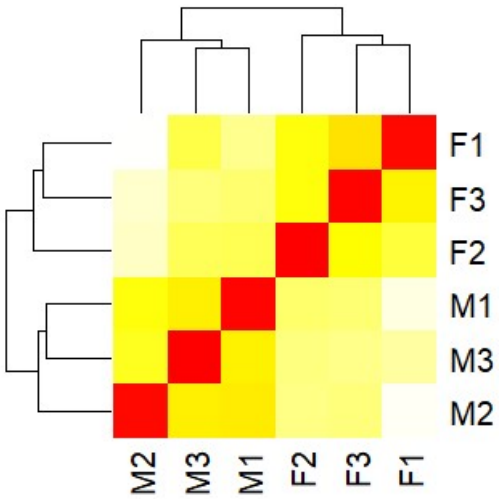
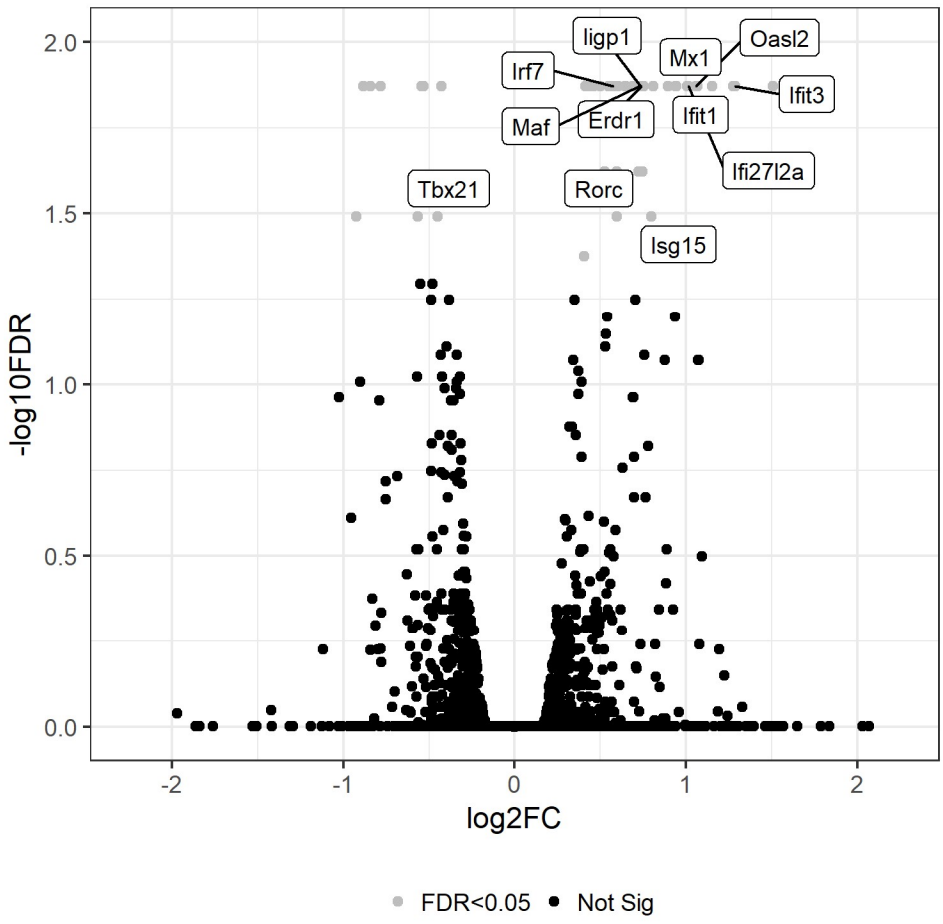

F1
F3
F2
M1
M3
M2
M2
M3
M1
F2
F3
F1

Figure 1 (continued)

C.



GO analysis was performed to look at enriched pathways and functions using GOrilla.[87,94] Redundant GO terms were compressed into overarching categories using REViGO (Figure 2).[88] Out of the three GO aspects (molecular function, biological process, and cellular component), enriched categories were only found in biological processes. The enriched categories from this analysis further demonstrate the connection between the upregulated genes in naïve female $CD8^+$ T-cells and the response to viral antigens normally mediated by Type 1 IFNs. Two of the categories, “cellular response to

interferon-alpha” and “response to interferon-beta,” refer to gene sets directly stimulated by 2 types of Type 1 IFNs. Other categories, such as “defense response to virus,” “negative regulation of viral genome replication,” and “response to biotic stimulus,” show differential enrichment of viral response genes.

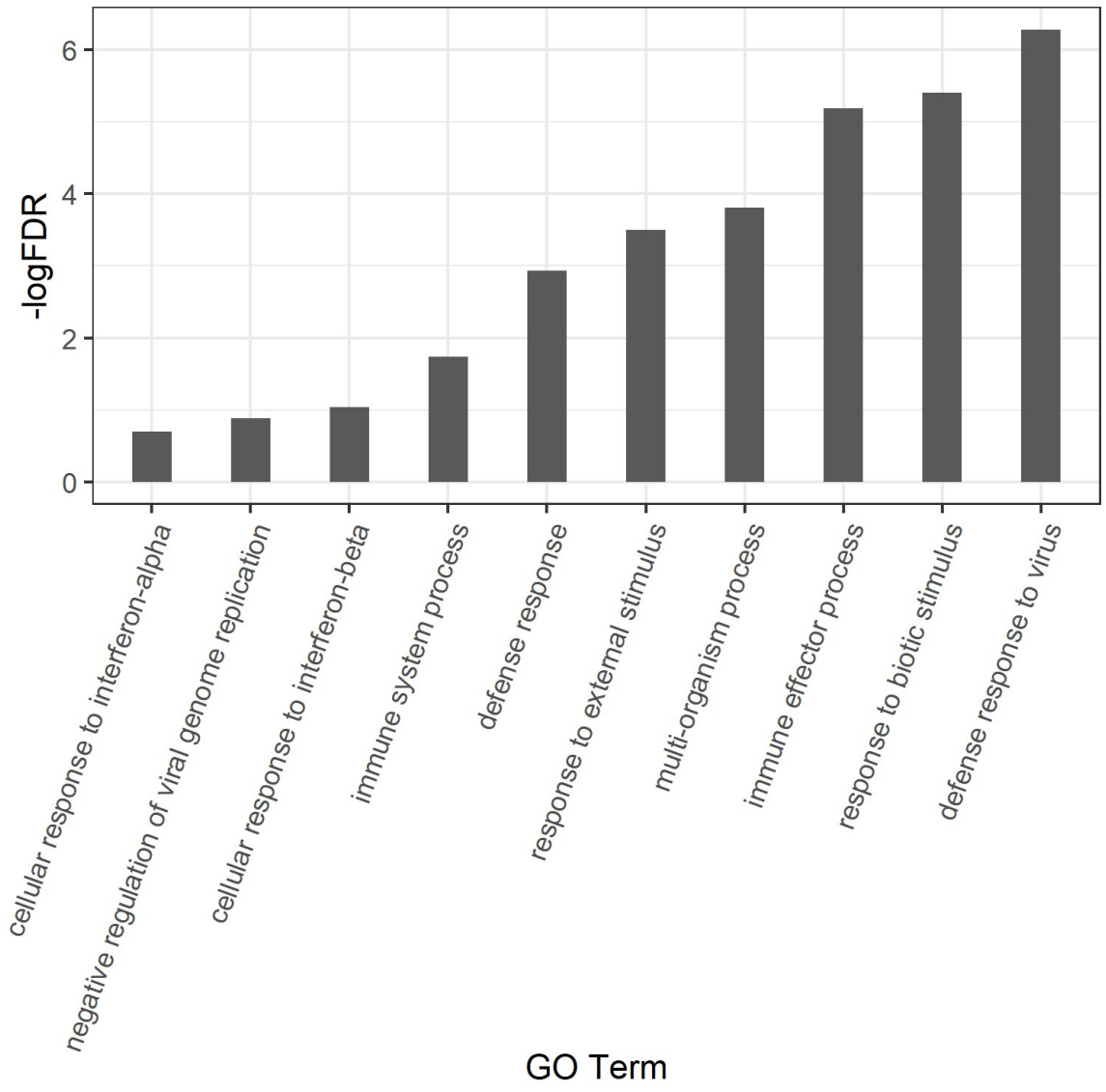


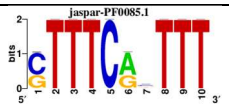
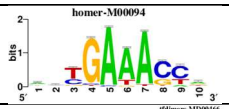
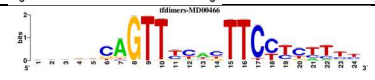
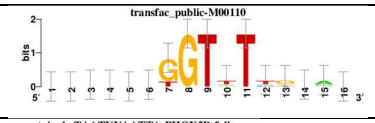
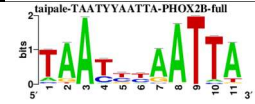
Figure 2: GO biological process enrichment analysis of genes upregulated in female naïve CD8⁺ T-cells. Redundant GO terms have been filtered out using REViGO (original categories=23). All categories enriched at an FDR<0.25 are shown.

Sex specific differences between naïve CD8⁺ T-cells can be linked to specific transcription factor motifs

To further investigate the pathways involved in the sex specific differences between naïve CD8⁺ T-cells, motif enrichment analysis was performed to search for transcription factors that could be modulating gene expression in males versus females through binding near gene promoters.[91]

Interestingly, this analysis showed enrichment for motifs from several transcription factors related to interferon responses (Table 1). The top 3 enriched motif clusters all contain motifs for interferon response factors (IRFs), transcription factors known to alter gene expression in cells upon stimulation with interferons.[95]

Table 1: Top 5 enriched motif clusters for differentially expressed genes in naïve $CD8^+$ T-cells. The search for motifs was restricted from -500bp to +500bp around the transcription start site of each gene. NES= normalized enrichment score. #T= number of gene targets containing motifs from a specific motif cluster.

Motif Cluster	Representative Motif ID	Logo	Associated TFs	NES	#T
M1	jaspar-PF0085.1		Irf2, Irf7, Irf8, Irf6, Irf3, Irf4, Irf1, Irf5, Irf9, E2f1, Myb, Zfp683, Stat1, Stat2	8.25	16
M3	homer-M00094		Irf5, Irf6, Irf4, Irf3	5.29	13
M4	tfdimers-MD00466		Myb, Sfpi1, Irf6, Irf2, Irf8, Irf1, Irf4, Irf3, Irf7, Irf5, Prdm1, Elf1, E2f1, Pura	5.18	8
M7	transfac_public-M00110		Grhl2, Grhl1, Grhl3	4.03	4
M8	taipale-TAATYYAATTA_PHOX2B_FULL		Phox2b, Hoxd8, Hoxb8, Phox2a, Uncx, Prrxl1, Alx4, Isx, Pitx3, Dbp, Crx	3.96	4

While the binding motifs of these IRFs are difficult to deconvolute due to their sequence similarity, cross-referencing the candidate IRFs with the RNA-seq data shows that the only sex specific IRF is IRF7, which is upregulated in

the female naïve CD8$^+$ T-cells. IRF7, which is normally activated by pattern recognition receptor signaling in innate immune cells, is one of the master regulators of the Type I IFN response.[96] IRF7 directly modulates several critical members of the Type I IFN response that are differentially expressed between males and females, including the Ifit family members Ifit1, Ifit3, and Ifi27l2a, Mx1, and Isg15 (Figure 3).

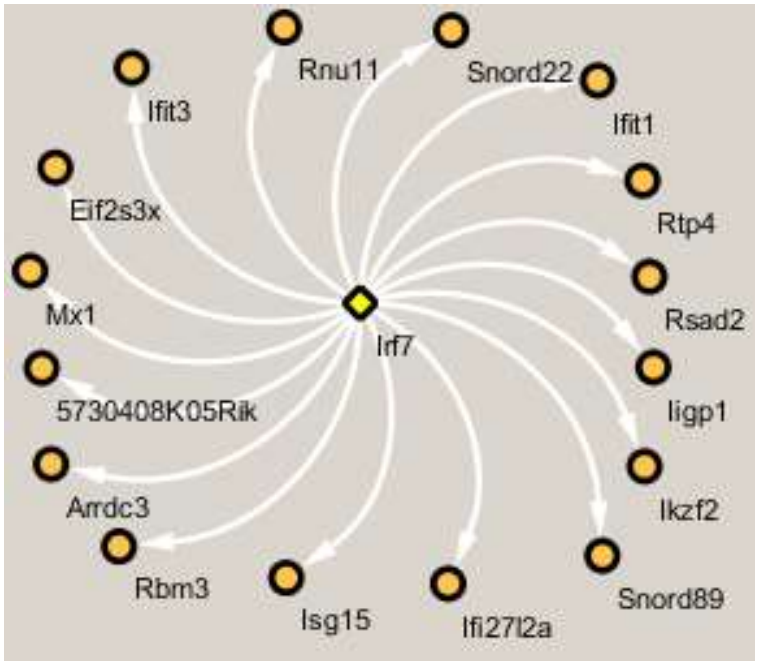


Figure 3: Differentially expressed genes between the sexes that are regulated by IRF7, based on the results from iRegulon motif enrichment analysis. Notable immune regulators upregulated by IRF7 include several members of the Ifit family, Mx1, Isg15, Rsad2, and Iigp1.

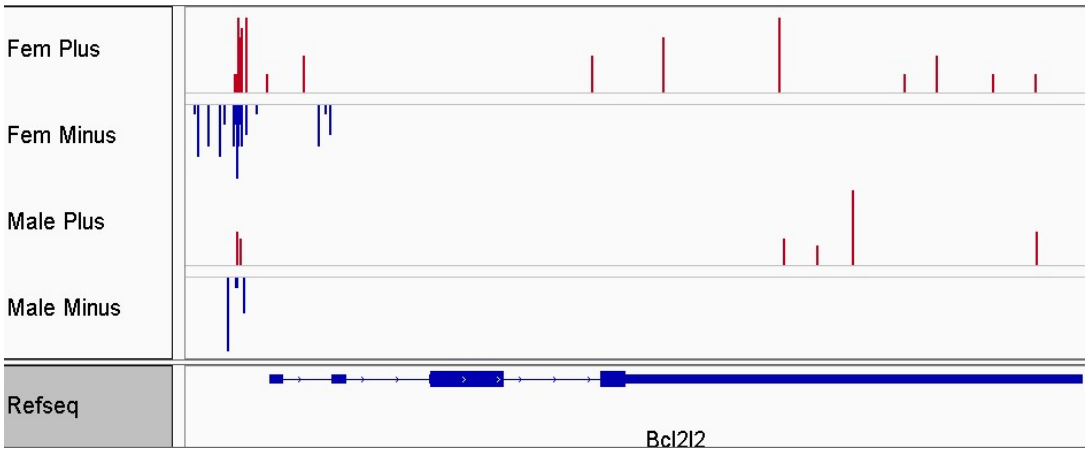
PRO-seq shows sex specific differential expression at promoters

RNA-seq provides information about gene expression, but it does not help to identify areas in the genome that may potentially be acting as transcriptional regulatory elements. So, in addition to RNA-seq, PRO-seq was

performed on naïve $CD8^+$ T-cells purified via magnetic bead enrichment for CD8 from n=1 females and n=2 males (Figure 4). PRO-seq allows for the evaluation of transcriptional regulatory elements (e.g. promoters, enhancers) by identifying areas of divergent transcription. Additionally, the reads from gene bodies can be used to gain information about gene expression in a manner similar to RNA-seq. Combining these two classes of information can reveal promoters that differ in their levels of RNA Polymerase II occupancy but are not in genes being actively transcribed. While the RNA-seq results revealed a small number of differentially expressed genes between naïve male and female $CD8^+$ T-cells, it is also possible that there is a set of genes poised for activation upon antigen stimulation that differs between the sexes (Figure 4a). In order to test this possibility, we evaluated the subset of genes that have sex differential amounts of reads in their promoter regions (here defined as -1000bp, +500bp from the TSS), but are not differentially expressed according to both the gene body reads from PRO-seq and the RNA-seq data (Figure 4b). 468 promoters were discovered to be differentially occupied between males and females. However, it is important to note that the lack of replicates for the female samples means that this analysis only constitutes very preliminary results.

Figure 4: Differential occupancy of potentially poised promoters. **A)** A genome browser shot showing an example of a gene on chromosome 14 (Bcl2l2) with larger amounts of reads at its promoter in naïve female $CD8^+$ T-cells, but which is not differentially expressed according to PRO-seq and RNA-seq results. Note the divergent transcription that marks the promoter region as a transcriptional regulatory element. **B)** Table describing the number of differential promoters, differentially expressed genes, and differential promoters from non-differentially expressed genes. **C)** Principal component analysis performed on the log transformed counts from the promoters.

A.

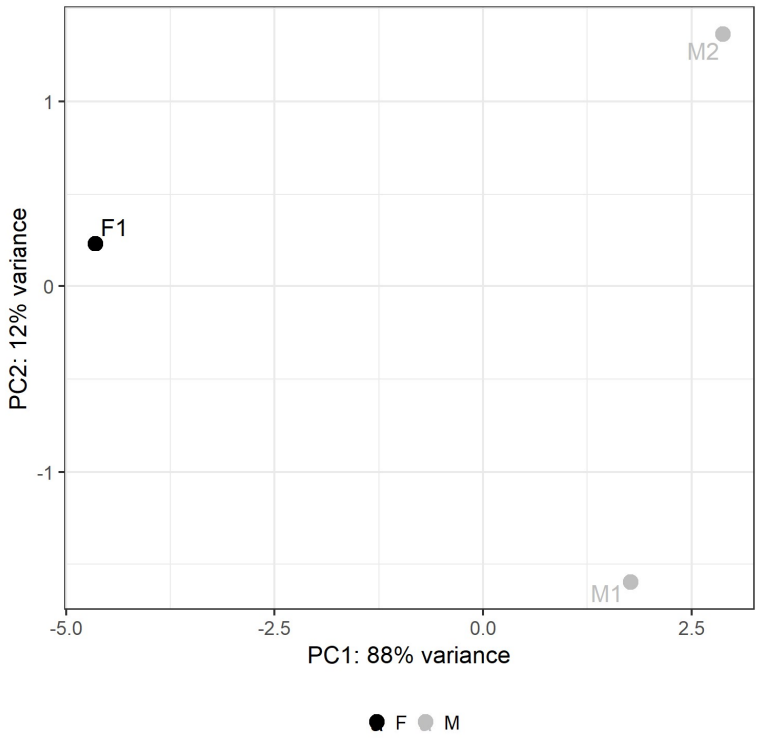


B.

Differentially Expressed Genes (from PRO-seq and RNA-seq)	450
Differential Promoters (from PRO-seq)	493
Differential Promoters from Non-Differentially Expressed Genes	468

Figure 4 (continued)

c.



PCA shows a divide between the global reads at promoters in the male and female samples (Figure 4c). GO analysis using these promoters showed no statistically significant (FDR ≤ 0.25) enriched categories for any GO aspect (Figure 5). It is possible that there are no significant overarching signaling pathways controlling the differences between the promoters, or that more replicates are required to discover potential differences. However, it is interesting to note that while none of the GO enrichment categories found by GOrilla clear the set significance threshold, the categories called enriched are still consistent with alterations in the Type I IFN response pathway (e.g. “defense response,” “regulation of defense response to virus by host,”

“regulation of CD4-positive, alpha-beta T cell differentiation”) (Figure 5A). In addition to the GO enrichment analysis, GSEA was performed to compare the differential promoters to $CD8^+$ T-cell gene signatures from the Molecular Signatures Database (MSigDB), as well as gene clusters from the Immunological Genome Project (ImmGen) Consortium.[89,90] A subset of eight gene lists from the MSigDB C7 immunological signatures related to SLEC vs MPEC gene expression was used, and one of these gene sets describing genes that are downregulated in effector cells vs memory cells was found to be enriched in the promoters that are more highly transcribed in males (Figure 5B). This finding is consistent with previous data suggesting that naïve male $CD8^+$ T-cells form a higher ratio of MPECs vs SLECs. The promoters that have higher amounts of reads in males also show some correlation with Cluster V from the ImmGen $CD8^+$ T-cell data. (Figure 5C). This cluster is enriched for genes that are activated in naïve, early activated, or memory T-cells, as opposed to other clusters enriched for effector response genes. This may hint to a possible readiness for expression of memory phenotype genes in the males, but notably none of the clusters or MSigDB gene sets were enriched in the upregulated female promoters.

Figure 5: GO enrichment analysis and GSEA results for promoters detected by PRO-seq. **A)** GO biological process enrichment analysis of promoters changed in naïve $CD8^+$ T-cells. Redundant GO terms have been filtered out using REViGO (original categories=21). Note that none of the presented categories pass an FDR cutoff of FDR ≤ 0.25. **B)** GSEA enrichment plot for the differential promoters from non-differentially expressed genes compared to genes downregulated in effector vs. memory $CD8^+$ T-cells. “na_pos” (red) refers to promoters expressed more highly in naïve female $CD8^+$ T-cells, while “na_neg” (blue) refers to promoters that are downregulated in females/upregulated in males. **C)** GSEA enrichment plot for the differential promoters from non-differentially expressed genes compared to Cluster V from Best et al. 2013. “na_pos” (red) refers to promoters expressed more highly in naïve female $CD8^+$ T-cells, while “na_neg” (blue) refers to promoters that are downregulated in females/upregulated in males.

A.

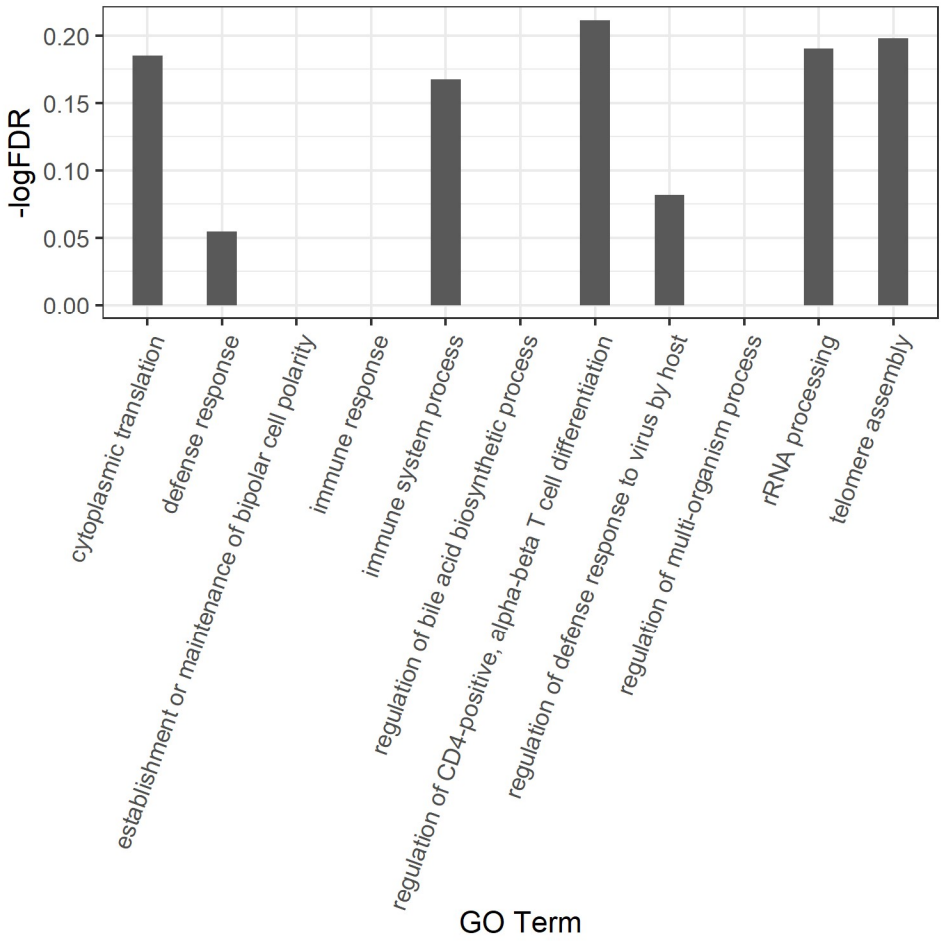

-logFDR
0.20
0.15
0.10
0.05
0.00
cytoplasmic translation
defense response
establishment or maintenance of bipolar cell polarity
immune response
immune system process
regulation of bile acid biosynthetic process
regulation of CD4-positive, alpha-beta T cell differentiation
regulation of defense response to virus by host
regulation of multi-organism process
rRNA processing
telomere assembly
GO Term

Figure 5 (continued)

B.

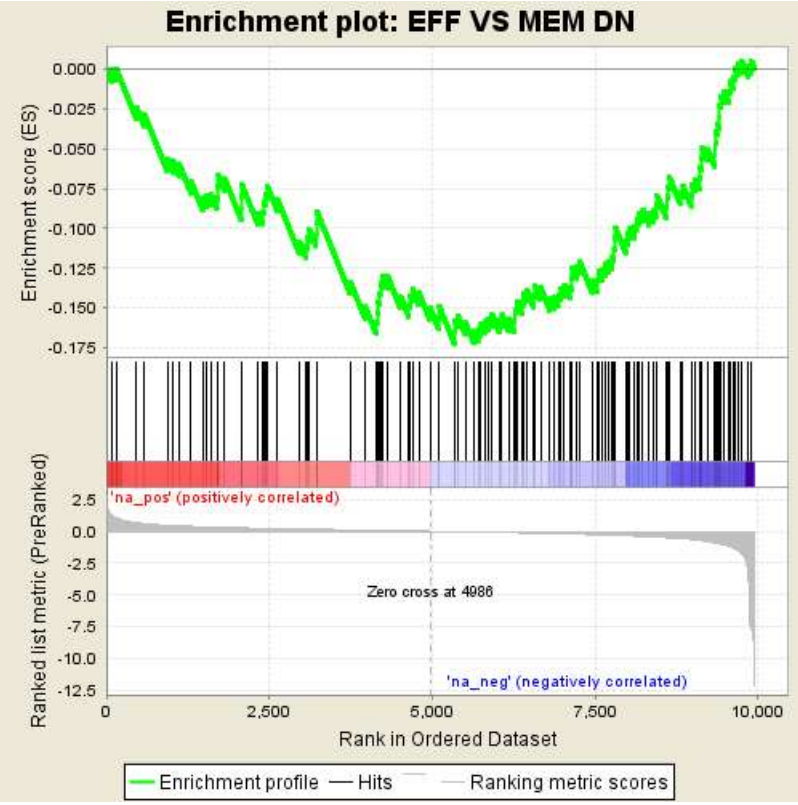
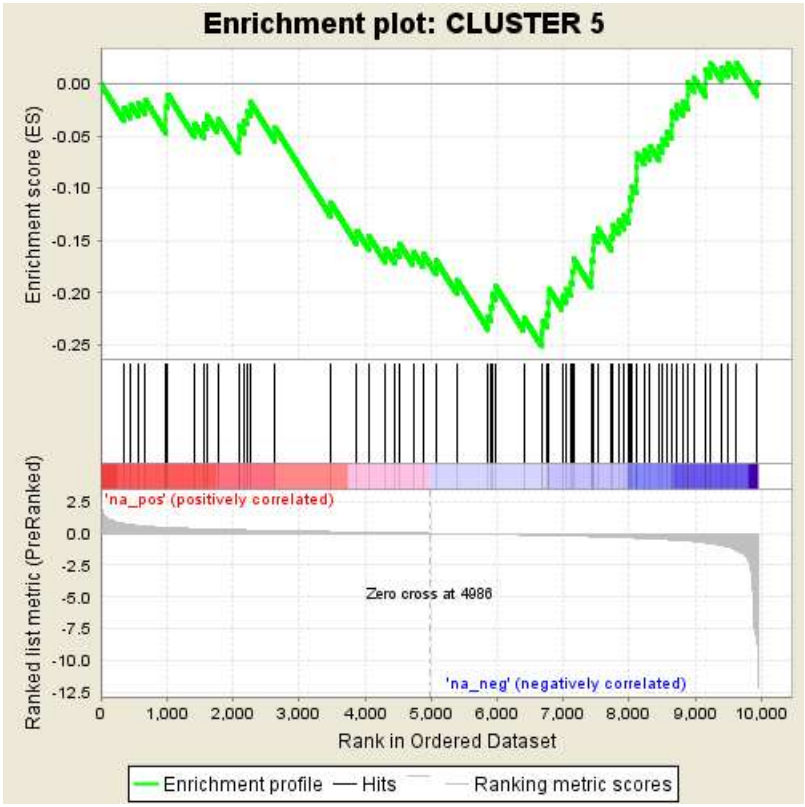


Figure 5 (continued)

C.



Discussion

Experiments described herein suggest a potential role for expression of Type 1 IFN response genes in the sex specific differences between naïve male and female $CD8^+$ T-cells. This is not surprising, considering the fact that the Type 1 IFN response has been implicated in many autoimmune disorders that are more prevalent in females. For example, the aberrant production and activation of Type 1 IFNs through pattern recognition receptors plays a critical role in the pathogenesis of SLE.[97,98] Expression of a particular haplotype of IRF5 is associated with a higher chance of developing SLE.[99,100] Blocking expression of Type 1 IFNs can prevent the development of arthritis in mice with long term Lyme disease.[101] More generally, IFNα administered to cancer patients causes an increased incidence of autoimmune disorders (e.g. SLE, autoimmune vasculitis, thyroid disease), especially in females.[102] Additionally, Type I IFNs can influence the formation of SLECs versus MPECs after antigen exposure. Type I IFNs are required for the development of SLECs in mice, and their loss leads to the formation of an effector cell population dominated by MPECs.[103]

Type 1 IFNs initiate a complex signal cascade through a shared heterodimeric receptor, IFNAR.[104] Activation of this receptor causes localization of a STAT complex to the nucleus, which in turn activates ~200 interferon response genes.[105] While the STAT complex was first described as a STAT1:STAT2 dimer, there are 5 other STAT family members that can also modulate the downstream response.[105,106] Several factors from this pathway

were upregulated in naïve female $CD8^+$ T-cells (fold change=1.5-2.8), many of which are involved in recognizing viral RNA. The Ifit family plays an important role in the recognition of viral RNA, binding to 5' triphosphate ends or 5' caps lacking 2′-O-methylation marks that would normally be present on cellular caps.[93,107] This binding activity, along with interactions between the Ifits and eIF3c (a subunit of the translation preinitiation complex), helps prevent the translation of viral RNAs.[108] Ifit1 and Ifit2 are known to be highly expressed in T-cells.[109] Ifit3 and Ifit4 have been tied to the development of autoimmunity in mice, and Ifit3 is upregulated in females (fold change=2.4).[110,111] Mx proteins also help to defend against RNA viruses through a variety of transcriptional inhibition mechanisms, and their deletion leaves mice highly susceptible to influenza.[112] The protein Oasl2 specializes in recognizing double-stranded RNA, and overexpression of Oasl2 in mice leads to inhibition of viral replication.[113,114]

Interferon stimulated gene 15 (Isg15), a ubiquitin-like modifier, is also upregulated in female $CD8^+$ T-cells with a fold change of 1.7.[115,116] Isg15 can act as a cytokine, and can also be attached to both host and viral proteins in a process called ISGylation.[116] As a cytokine, Isg15 can stimulate the production of IFNγ and attract cells from the innate immune response to dispose of cell debris.[116] ISGylation serves as its main mechanism in intracellular signaling, allowing Isg15 to downregulate viral replication as well as tweak host translation and exosome secretion.[115] Interestingly, while the deletion of Isg15

in mice causes increased susceptibility to viral infection and decreased Type I IFN responses, the opposite is true for humans who are lacking ISG15.[117]

Interferon response factor 7 (IRF7) is required for robust activation of the Type I IFN response.[96] IRF7 is expressed in an inactive form in the cytoplasm and then phosphorylated after activation of pattern recognition receptors.[118,119] Along with its potential binding partners IRF3 and NFκB, it enters the nucleus and induces Type I IFN production. This increased production leads to the release of Type I IFNs, which then activate the IFNARs on other cells.[120] IRF7 is expressed ~1.5 times more highly in the naïve female $CD8^+$ T-cells, and its binding motif is also highly enriched in the differentially expressed genes from the RNA-seq experiment. This brings up the possibility that higher basal expression of IRF7 in naïve female $CD8^+$ T-cells allows for a more rapid and/or easily induced response to Type I IFNs in the female cells. Previous work has suggested that knocking out IRF7 in mouse $CD8^+$ T-cells can reduce the number of effector T-cells specific for a particular antigen, but this connection to effector T-cell function must be more extensively investigated in the future.[121]

While the evidence presented thus far supports the hypothesis that type I IFN signaling is stronger in naïve female $CD8^+$ T-cells than in naïve male $CD8^+$ T-cells and that this signaling may be related to the increased SLEC:MPEC ratio in females, it is important to note the single immune regulator upregulated in males versus females: Tbx21, the T-box gene encoding for the transcription factor T-bet. T-bet's role in $CD8^+$ T-cell

differentiation has been studied extensively, and it has been demonstrated that higher levels of T-bet push T-cells towards differentiating into SLECs.[122] T-bet's function as an amplifier of Type I IFN responses seems out of place in the male naïve $CD8^+$ T-cells. However, it has been shown in $CD4^+$ T-cells exposed to high levels of IFNγ that T-bet can act as a suppressor of the Type 1 IFN response, indicating that T-bet's exact role in effector cell differentiation is highly context dependent.[123] It is possible that in naïve male $CD8^+$ T-cells, T-bet may somehow be suppressing Type I IFN signaling. Alternately, the higher levels of T-bet in the male $CD8^+$ T-cells may not be enough to have a noticeable effect on IFN signaling in the male cells.

The concept of differentially poised promoters in the sexes is intriguing, but unfortunately the currently available data is unable to strongly support or deny this hypothesis. PRO-seq experiments using a higher number of replicates would be useful in further evaluating transcriptional regulatory elements. ATAC-seq, which can detect open regions of chromatin, can also be used to look at genomic regions that differ between the sexes in naïve $CD8^+$ T-cells.[124] Additional experiments in mice involving knockdowns of the Type I IFN receptor or Irf7 may show that lowering the signal from this pathway can cause female $CD8^+$ T-cells to behave more like their male counterparts, while the reverse may be true for male cells where these proteins are upregulated. Overall, the data presented here suggest that the Type I IFN response pathway may control the sex specific differences in naïve $CD8^+$ T-cell expansion and differentiation, and further investigations into the effects of Irf7

expression on T-cell differentiation would help to expand our understanding of autoimmunity.

Literature Cited

1. Vivier, E., Pavert, S. A. van de, Cooper, M. D. & Belz, G. T. The evolution of innate lymphoid cells. *Nature Immunology* **17,** 790–794 (2016).
2. Janeway, C. A. Approaching the Asymptote? Evolution and Revolution in Immunology. *Cold Spring Harb Symp Quant Biol* **54,** 1–13 (1989).
3. Burnet, F. M. *The clonal selection theory of acquired immunity.* (Vanderbilt University Press, 1959).
4. Cooper, M. D. & Alder, M. N. The Evolution of Adaptive Immune Systems. *Cell* **124,** 815–822 (2006).
5. Gasteiger, G. & Rudensky, A. Y. Interactions between innate and adaptive lymphocytes. *Nature Reviews Immunology* **14,** 631–639 (2014).
6. MacDonald, H. R., Radtke, F. & Wilson, A. T cell fate specification and αβ/γδ lineage commitment. *Current Opinion in Immunology* **13,** 219–224 (2001).
7. Condotta, S. A. & Richer, M. J. The immune battlefield: The impact of inflammatory cytokines on CD8+ T-cell immunity. *PLOS Pathogens* **13,** e1006618 (2017).
8. Oldstone, M. B. A. & Dixon, F. J. Tissue injury in lymphocytic choriomeningitis viral infection: Virus-induced immunologically specific release of a cytotoxic factor from immune lymphoid cells. *Virology* **42,** 805–813 (1970).
9. Pamer, E. & Cresswell, P. Mechanisms of mhc class i–restricted antigen processing. *Annu. Rev. Immunol.* **16,** 323–358 (1998).
10. Boise, L. H. *et al.* CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xL. *Immunity* **3,** 87–98 (1995).
11. Carreno, B. M. & Collins, M. The B7 Family of Ligands and Its Receptors: New Pathways for Costimulation and Inhibition of Immune Responses. *Annu. Rev. Immunol.* **20,** 29–53 (2002).
12. Curtsinger, J. M. & Mescher, M. F. Inflammatory Cytokines as a Third Signal for T Cell Activation. *Curr Opin Immunol* **22,** 333–340 (2010).
13. Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302,** 575–581 (1983).
14. Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: what thymocytes see and don't see. *Nat Rev Immunol* **14,** 377–391 (2014).
15. Valbon, S. F., Condotta, S. A. & Richer, M. J. Regulation of effector and memory CD8+ T cell function by inflammatory cytokines. *Cytokine* **82,** 16–23 (2016).
16. Butz, E. A. & Bevan, M. J. Massive Expansion of Antigen-Specific CD8+ T Cells during an Acute Virus Infection. *Immunity* **8,** 167–175 (1998).
17. Kaech, S. M., Wherry, E. J. & Ahmed, R. Vaccines: Effector and memory T-cell differentiation: implications for vaccine development. *Nature Reviews Immunology* **2,** 251–262 (2002).
18. Williams, M. A. & Bevan, M. J. Effector and Memory CTL Differentiation. *Annual Review of Immunology* **25,** 171–192 (2007).
19. Goldrath, A. W., Bogatzki, L. Y. & Bevan, M. J. Naive T Cells Transiently Acquire a Memory-like Phenotype during Homeostasis-Driven Proliferation. *Journal of Experimental Medicine* **192,** 557–564 (2000).
20. Akue, A. D., Lee, J.-Y. & Jameson, S. C. Derivation and Maintenance of Virtual Memory CD8 T Cells. *The Journal of Immunology* **188,** 2516–2523 (2012).
21. Parish, I. A. & Kaech, S. M. Diversity in CD8+ T cell differentiation. *Current Opinion in Immunology* **21,** 291–297 (2009).

22. Kaech, S. M. *et al.* Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nature Immunology* **4,** 1191–1198 (2003).
23. Henson, S. M. & Akbar, A. N. KLRG1—more than a marker for T cell senescence. *Age (Dordr)* **31,** 285–291 (2009).
24. Lefrançois, L. & Obar, J. J. Once a killer, always a killer: from cytotoxic T cell to memory cell. *Immunological Reviews* **235,** 206–218
25. Sarkar, S. *et al.* Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *Journal of Experimental Medicine* **205,** 625–640 (2008).
26. Kaech, S. M. & Wherry, E. J. Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection. *Immunity* **27,** 393–405 (2007).
27. Obar, J. J. *et al.* CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *PNAS* **107,** 193–198 (2010).
28. Smith, N. L. *et al.* Rapid proliferation and differentiation impairs the development of memory CD8+ T cells in early life. *Journal of immunology (Baltimore, Md. : 1950)* **193,** 177–84 (2014).
29. Wissink, E. M., Smith, N. L., Spektor, R., Rudd, B. D. & Grimson, A. MicroRNAs and their targets are differentially regulated in adult and neonatal mouse CD8+ T Cells. *Genetics* **201,** 1017–1030 (2015).
30. Smith, N. L., Wissink, E. M., Grimson, A. & Rudd, B. D. miR-150 Regulates Differentiation and Cytolytic Effector Function in CD8+ T cells. *Scientific Reports* **5,** 16399 (2015).
31. Wang, J. *et al.* Fetal and adult progenitors give rise to unique populations of CD8+ T cells. *Blood* **128,** 3073–3082 (2016).
32. Watson, N. B. *et al.* Neonatal CD8+ T cells provide innate immune protection against unrelated pathogens. *The Journal of Immunology* **198,** 151.3-151.3 (2017).
33. Smith, N. L. *et al.* Developmental Origin Governs CD8+ T Cell Fate Decisions during Infection. *Cell* **174,** 117-130.e14 (2018).
34. Wang, J. *et al.* Fetal and adult progenitors give rise to unique populations of CD8+ T cells. *Blood* **128,** (2016).
35. White, J. T. *et al.* Virtual memory T cells develop and mediate bystander protective immunity in an IL-15-dependent manner. *Nat Commun* **7,** (2016).
36. Liang, Y., Pan, H.-F. & Ye, D.-Q. microRNAs function in CD8+T cell biology. *Journal of Leukocyte Biology* **97,** 487–497
37. Bupp, G., R, M., Potluri, T., Fink, A. L. & Klein, S. L. The Confluence of Sex Hormones and Aging on Immunity. *Front. Immunol.* **9,** (2018).
38. Steeg, L. G. vom & Klein, S. L. SeXX Matters in Infectious Disease Pathogenesis. *PLOS Pathogens* **12,** e1005374 (2016).
39. Klein, S. L. & Flanagan, K. L. Sex differences in immune responses. *Nature Reviews Immunology* **16,** 626–638 (2016).
40. Whitacre, C. C. Sex differences in autoimmune disease. *Nature Immunology* (2001). doi:10.1038/ni0901-777
41. Klein, S. L. Immune Cells Have Sex and So Should Journal Articles. *Endocrinology* **153,** 2544–2550 (2012).
42. Hewagama, A. *et al.* Overexpression of X-Linked Genes in T Cells From Women With Lupus. *J Autoimmun* **41,** 60–71 (2013).

43. Baltimore, D., Boldin, M. P., O'Connell, R. M., Rao, D. S. & Taganov, K. D. MicroRNAs: new regulators of immune cell development and function. *Nature Immunology* **9,** 839–845 (2008).
44. Capone, I., Marchetti, P., Ascierto, P. A., Malorni, W. & Gabriele, L. Sexual Dimorphism of Immune Responses: A New Perspective in Cancer Immunotherapy. *Front. Immunol.* **9,** (2018).
45. Hewagama, A., Patel, D., Yarlagadda, S., Strickland, F. M. & Richardson, B. C. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. *Genes and Immunity* **10,** 509–516 (2009).
46. Zein, J. G. & Erzurum, S. C. Asthma is Different in Women. *Curr Allergy Asthma Rep* **15,** 28 (2015).
47. Walsh, S. J. & Rau, L. M. Autoimmune diseases: a leading cause of death among young and middle-aged women in the United States. *Am J Public Health* **90,** 1463–1466 (2000).
48. Wilder, R. L. Hormones, Pregnancy, and Autoimmune Diseases. *Annals of the New York Academy of Sciences* **840,** 45–50
49. Poppe, K., Velkeniers, B. & Glinoer, D. Thyroid disease and female reproduction. *Clinical Endocrinology* **66,** 309–321
50. Glintborg, D. & Andersen, M. Medical comorbidity in polycystic ovary syndrome with special focus on cardiometabolic, autoimmune, hepatic and cancer diseases: an updated review. *Current Opinion in Obstetrics and Gynecology* **29,** 390 (2017).
51. Shoenfeld, Y., Selmi, C., Zimlichman, E. & Gershwin, M. E. The autoimmunologist: geoepidemiology, a new center of gravity, and prime time for autoimmunity. *Journal of Autoimmunity* **31,** 325–330 (2008).
52. Phiel, K. L., Henderson, R. A., Adelman, S. J. & Elloso, M. M. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunology Letters* **97,** 107–113 (2005).
53. Straub, R. H. The Complex Role of Estrogens in Inflammation. *Endocr Rev* **28,** 521–574 (2007).
54. Fox, H. S., Bond, B. L. & Parslow, T. G. Estrogen regulates the IFN-gamma promoter. *The Journal of Immunology* **146,** 4362–4367 (1991).
55. Anderson, M. S. *et al.* Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298,** 1395–1401 (2002).
56. Dragin, N. *et al.* Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. *J Clin Invest* **126,** 1525–1537
57. Menzies, F. M., Henriquez, F. L., Alexander, J. & Roberts, C. W. Selective inhibition and augmentation of alternative macrophage activation by progesterone. *Immunology* **134,** 281–291
58. Yang, F., Babak, T., Shendure, J. & Disteche, C. M. Global survey of escape from X inactivation by RNA-sequencing in mouse. *Genome Res* **20,** 614–622 (2010).
59. Fish, E. N. The X-files in immunity: sex-based differences predispose immune responses. *Nature Reviews Immunology* **8,** 737–744 (2008).
60. Cacciari, E. *et al.* Serum Immunoglobulins and Lymphocyte Subpopulations Derangement in Turner's Syndrome. *International Journal of Immunogenetics* **8,** 337–344
61. Cooney, C. M. *et al.* 46,X,del(X)(q13) Turner's Syndrome Female with Systemic Lupus Erythematosus in a Pedigree Multiplex for SLE. *Genes Immun* **10,** 478–481 (2009).
62. Scofield, R. H. *et al.* Klinefelter's Syndrome, 47,XXY, in Male Systemic Lupus Erythematosus Supports a Gene Dose Effect from the X Chromosome. *Arthritis Rheum* **58,** 2511–2517 (2008).

63. Koturbash, I., Zemp, F., Kolb, B. & Kovalchuk, O. Sex-specific radiation-induced microRNAome responses in the hippocampus, cerebellum and frontal cortex in a mouse model. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **722,** 114–118 (2011).
64. Hekim, N., Cetin, Z., Nikitaki, Z., Cort, A. & Saygili, E. I. Radiation triggering immune response and inflammation. *Cancer Letters* **368,** 156–163 (2015).
65. Dai, R. *et al.* Sex differences in the expression of lupus-associated miRNAs in splenocytes from lupus-prone NZB/WF1 mice. *Biology of Sex Differences* **4,** 19 (2013).
66. Mihelich, B. L. *et al.* miR-183-96-182 Cluster Is Overexpressed in Prostate Tissue and Regulates Zinc Homeostasis in Prostate Cells. *J. Biol. Chem.* **286,** 44503–44511 (2011).
67. Valastyan, S. & Weinberg, R. A. miR-31: A crucial overseer of tumor metastasis and other emerging roles. *Cell Cycle* **9,** 2124–2129 (2010).
68. Xue, F. *et al.* miR-31 regulates interleukin 2 and kinase suppressor of ras 2 during T cell activation. *Genes and Immunity* **14,** 127–131 (2013).
69. Chen, J., Wang, M., Guo, M., Xie, Y. & Cong, Y.-S. miR-127 Regulates Cell Proliferation and Senescence by Targeting BCL6. *PLOS ONE* **8,** e80266 (2013).
70. Tam, W., Ben-Yehuda, D. & Hayward, W. S. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol Cell Biol* **17,** 1490–1502 (1997).
71. Gracias, D. T. *et al.* The microRNA miR-155 controls CD8+ T cell responses by regulating interferon signaling. *Nature Immunology* **14,** 593–602 (2013).
72. Dudda, J. C. *et al.* MicroRNA-155 is required for effector CD8+ T cell responses to virus infection and cancer. *Immunity* **38,** 742–53 (2013).
73. Laird, S. M. *et al.* A review of immune cells and molecules in women with recurrent miscarriage. *Hum Reprod Update* **9,** 163–174 (2003).
74. Zuk, M. Reproductive strategies and disease susceptibility: an evolutionary viewpoint. *Parasitology Today* **6,** 231–233 (1990).
75. Stoehr, A. M. & Kokko, H. Sexual dimorphism in immunocompetence: what does life-history theory predict? *Behav Ecol* **17,** 751–756 (2006).
76. Fink, A. L. & Klein, S. L. The evolution of greater humoral immunity in females than males: implications for vaccine efficacy. *Current Opinion in Physiology* **6,** 16–20 (2018).
77. Kim, D. *et al.* TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology* **14,** R36 (2013).
78. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28,** 511–515 (2010).
79. Trapnell, C. *et al.* Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnology* **31,** 46–53 (2013).
80. Mahat, D. B. *et al.* Base-pair-resolution genome-wide mapping of active RNA polymerases using precision nuclear run-on (PRO-seq). *Nature Protocols* **11,** 1455–1476 (2016).
81. Kwak, H., Fuda, N. J., Core, L. J. & Lis, J. T. Precise Maps of RNA Polymerase Reveal How Promoters Direct Initiation and Pausing. *Science* **339,** 950–953 (2013).
82. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17,** 10–12 (2011).

83. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10,** R25 (2009).
84. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30,** 923–930 (2014).
85. Danko, C. G. *et al.* Identification of active transcriptional regulatory elements from GRO-seq data. *Nature Methods* **12,** 433–438 (2015).
86. McCarthy, D. J., Chen, Y. & Smyth, G. K. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* **40,** 4288–4297 (2012).
87. Eden, E., Navon, R., Steinfeld, I., Lipson, D. & Yakhini, Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* **10,** 48 (2009).
88. Supek, F., Bošnjak, M., Škunca, N. & Šmuc, T. REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. *PLOS ONE* **6,** e21800 (2011).
89. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *PNAS* **102,** 15545–15550 (2005).
90. Best, J. A. *et al.* Transcriptional insights into the CD8+ T cell response to infection and memory T cell formation. *Nature Immunology* **14,** 404–412 (2013).
91. Verfaillie, A., Imrichova, H., Janky, R. & Aerts, S. iRegulon and i-cisTarget: Reconstructing Regulatory Networks Using Motif and Track Enrichment. *Current Protocols in Bioinformatics* **52,** 2.16.1-2.16.39
92. Shannon, P. *et al.* Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **13,** 2498–2504 (2003).
93. Fensterl, V. & Sen, G. C. Interferon-induced Ifit proteins: their role in viral pathogenesis. *Journal of virology* **89,** 2462–8 (2015).
94. Eden, E., Lipson, D., Yogev, S. & Yakhini, Z. Discovering Motifs in Ranked Lists of DNA Sequences. *PLOS Computational Biology* **3,** e39 (2007).
95. Nguyen, H., Hiscott, J. & Pitha, P. M. The growing family of interferon regulatory factors. *Cytokine & Growth Factor Reviews* **8,** 293–312 (1997).
96. Ning, S., Pagano, J. S. & Barber, G. N. IRF7: activation, regulation, modification and function. *Genes and Immunity* **12,** 399–414 (2011).
97. Preble, O. T., Black, R. J., Friedman, R. M., Klippel, J. H. & Vilcek, J. Systemic Lupus Erythematosus: Presence in Human Serum of an Unusual Acid-Labile Leukocyte Interferon. *Science* **216,** 429–431 (1982).
98. Baccala, R., Hoebe, K., Kono, D. H., Beutler, B. & Theofilopoulos, A. N. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nature Medicine* **13,** 543–551 (2007).
99. Graham, R. R. *et al.* A common haplotype of interferon regulatory factor 5 (*IRF5*) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nature Genetics* **38,** 550–555 (2006).
100. Sigurdsson, S. *et al.* Polymorphisms in the Tyrosine Kinase 2 and Interferon Regulatory Factor 5 Genes Are Associated with Systemic Lupus Erythematosus. *The American Journal of Human Genetics* **76,** 528–537 (2005).
101. Miller, J. C. *et al.* A Critical Role for Type I IFN in Arthritis Development following Borrelia burgdorferi Infection of Mice. *The Journal of Immunology* **181,** 8492–8503 (2008).

102. Rönnblom, L. E., Alm, G. V. & Öberg, K. Autoimmune Phenomena in Patients with Malignant Carcinoid Tumors During Interferon-α Treatment. *Acta Oncologica* **30,** 537–540 (1991).
103. Wiesel, M. *et al.* Type-I IFN drives the differentiation of short-lived effector CD8+ T cells in vivo. *European Journal of Immunology* **42,** 320–329
104. Novick, D., Cohen, B. & Rubinstein, M. The human interferon αβ receptor: Characterization and molecular cloning. *Cell* **77,** 391–400 (1994).
105. Kessler, D. S., Levy, D. E. & Darnell, J. E. Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. *PNAS* **85,** 8521–8525 (1988).
106. Becerra-Díaz, M., Valderrama-Carvajal, H. & Terrazas, L. I. Signal Transducers and Activators of Transcription (STAT) Family Members in Helminth Infections. *Int J Biol Sci* **7,** 1371–1381 (2011).
107. Abbas, Y. M., Pichlmair, A., Górna, M. W., Superti-Furga, G. & Nagar, B. Structural basis for viral 5′-PPP-RNA recognition by human IFIT proteins. *Nature* **494,** 60–64 (2013).
108. Choi, Y. J., Bowman, J. W. & Jung, J. U. A Talented Duo: IFIT1 and IFIT3 Patrol Viral RNA Caps. *Immunity* **48,** 474–476 (2018).
109. Terenzi, F., White, C., Pal, S., Williams, B. R. G. & Sen, G. C. Tissue-specific and inducer-specific differential induction of ISG56 and ISG54 in mice. *Journal of virology* **81,** 8656–65 (2007).
110. Huang, X. *et al.* Interferon-induced protein IFIT4 is associated with systemic lupus erythematosus and promotes differentiation of monocytes into dendritic cell-like cells. *Arthritis Research & Therapy* **10,** R91 (2008).
111. Wang, J. *et al.* Elevated IFIT3 Contributes to Abnormal Overactive cGAS-STING Signaling in Human Systemic Lupus Erythematosus Monocytes. *Arthritis & Rheumatology* (2018). doi:10.1002/art.40576
112. Verhelst, J., Hulpiau, P. & Saelens, X. Mx Proteins: Antiviral Gatekeepers That Restrain the Uninvited. *Microbiol Mol Biol Rev* **77,** 551–566 (2013).
113. Eskildsen, S., Justesen, J., Schierup, M. H. & Hartmann, R. Characterization of the 2′–5′-oligoadenylate synthetase ubiquitin-like family. *Nucleic Acids Res* **31,** 3166–3173 (2003).
114. Bussey, K. A. *et al.* The interferon-stimulated gene product oligoadenylate synthetase-like protein enhances replication of Kaposi's sarcoma-associated herpesvirus (KSHV) and interacts with the KSHV ORF20 protein. *PLOS Pathogens* **14,** e1006937 (2018).
115. Villarroya-Beltri, C., Guerra, S. & Sánchez-Madrid, F. ISGylation - a key to lock the cell gates for preventing the spread of threats. *Journal of cell science* **130,** 2961–2969 (2017).
116. Perng, Y.-C. & Lenschow, D. J. ISG15 in antiviral immunity and beyond. *Nature Reviews Microbiology* **16,** 423–439 (2018).
117. Bogunovic, D. *et al.* Mycobacterial Disease and Impaired IFN-γ Immunity in Humans with Inherited ISG15 Deficiency. *Science* **337,** 1684–1688 (2012).
118. Wathelet, M. G. *et al.* Virus Infection Induces the Assembly of Coordinately Activated Transcription Factors on the IFN-β Enhancer In Vivo. *Molecular Cell* **1,** 507–518 (1998).
119. Barbalat, R., Lau, L., Locksley, R. M. & Barton, G. M. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nature Immunology* **10,** 1200–1207 (2009).

120. Marié, I., Durbin, J. E. & Levy, D. E. Differential viral induction of distinct interferon-α genes by positive feedback through interferon regulatory factor-7. *The EMBO Journal* **17,** 6660–6669 (1998).
121. Edwards, C. L. *et al.* Spatiotemporal requirements for IRF7 in mediating type I IFN-dependent susceptibility to blood-stage Plasmodium infection. *European Journal of Immunology* **45,** 130–141
122. Joshi, N. S. *et al.* Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. *Immunity* **27,** 281–295 (2007).
123. Shigeru Iwata. The Transcription Factor T-bet Limits Amplification of Type I IFN Transcriptome and Circuitry in T Helper 1 Cells. *Immunity* **46,** (2017).
124. Buenrostro, J. D., Wu, B., Chang, H. Y. & Greenleaf, W. J. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Current Protocols in Molecular Biology* **109,** 21.29.1-21.29.9 (2015).